Please type a sign(+) inside this box → ⊞

PTO/SB/05 rev 1 (12/97)

Approved for use through 09/30/00. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR § 1.53(b))

Attorney Docket No.	*1488.1040001/EKS/AJK*
First Named Inventor or Application Identifier	*Jing-Shan HU et al.*
Title	*Human Vascular Endothelial Growth Factor 3*
Express Mail Label No.	

03/03/98 C529 U.S. PTO

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

1. ☒ Fee Transmittal Form (*e.g.*, PTO/SB/17)
(Submit an original, and a duplicate for fee processing)

2. ☒ Specification [Total Pages 49]
(preferred arrangement set forth below)
- Descriptive title of the Invention
- Cross References to Related Applications
- Statement Regarding Fed sponsored R & D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings *(if filed)*
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

3. ☒ Drawing(s) (35 USC 113) [Total Sheets 2]

4. ☒ Oath or Declaration [Total Sheets 2]

a. ☐ Newly executed (original or copy)

b. ☒ Copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional with Box 17 completed)*
[Note Box 5 below]

i. ☐ DELETION OF INVENTOR(S)
Signed statement attachment deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).

5. ☒ Incorporation By Reference *(useable if Box 4b is checked)*
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. ☐ Microfiche Computer Program *(Appendix)*

7. Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all necessary)*

a. ☐ Computer Readable Copy

b. ☐ Paper Copy (identical to computer copy)

c. ☐ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & document(s))

9. ☐ 37 CFR 3.73(b) Statement (where there is an assignee) ☐ Power of Attorney

10. ☐ English Translation Document *(if applicable)*

11. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations

12. ☒ Preliminary Amendment

13. ☒ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)

14. ☐ Small Entity Statement(s) ☐ Statement filed in prior application, Status still proper and desired

15. ☐ Certified Copy of Priority Document(s)
(if foreign priority is claimed)

16. ☒ Other: 37 C.F.R. § 1.136(a)(3) Authorization to Treat a Reply as Incorporating an Extension of Time.

☒ Other: Second Preliminary Amendment

17. If a CONTINUING APPLICATION, *check appropriate box, and supply the requisite information below and in a preliminary amendment:*

☐ Continuation ☒ Divisional ☐ Continuation-in-part (CIP) of prior application No.: 08/469,641

18. CORRESPONDENCE ADDRESS

☐ *Customer Number or Bar Code Label* *(Insert Customer No. or Attach bar code label here)* *or* ☒ *Correspondence address below*

NAME	STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.				
	Attorneys at Law				
ADDRESS	Suite 600, 1100 New York Avenue, N.W.				
CITY	Washington	*STATE*	DC	*ZIP CODE*	20005-3934
COUNTRY	USA	*TELEPHONE*	(202) 371-2600	*FAX*	(202) 371-2540

NAME (Print/Type)	Eric K. Steffe	*Registration No. (Attorney/Agent)*	36,688
SIGNATURE		*Date* March 3, 1998	

Burden Hour Statement: this form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

PTO/SB/17 (12-97)
Approved for use through 08/30/00. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

FEE TRANSMITTAL

Note: Effective October 1, 1997,
Patent fees are subject to annual revision.

TOTAL AMOUNT OF PAYMENT	($) 790.00

Complete if Known	
Application Number	
Filing Date	March 3, 1998
First Named Inventor	Jing-Shan HU
Group Art Unit	To Be Assigned
Examiner Name	To Be Assigned
Attorney Docket Number	1488.1040001/EKS/AJK

METHOD OF PAYMENT

1. ☒ The Commissioner is hereby authorized to charge any deficiencies indicated fees and credit any overpayment to:

Deposit Account Number	19-0036
Deposit Name Account	Sterne, Kessler, Goldstein & Fox P.L.L.C.

☒ Charge Any Additional Fee Required Under 37 CFR 1.16 and 1.17 ☐ Charge the Issue Fee Set in 37 CFR 1.16 at the Mailing of the Notice of Allowance

2. ☒ Payment Enclosed:

☒ Check ☐ Money Order ☐ Other

FEE CALCULATION

1. FILING FEE

Large Fee Code	Entity Fee ($)	Small Fee Code	Entity Fee ($)	Fee Description	Fee Paid
101	790	201	395	Utility filing fee	$790.00
106	330	206	165	Design filing fee	
107	540	207	270	Plant filing fee	
108	790	208	395	Reissue filing fee	
114	150	214	75	Provisional filing fee	

SUBTOTAL (1) ($) _____

2. CLAIMS

		Extra	Fee from below	Fee Paid
Total Claims	20 -20 =	0	X _____ =	00.00
Indep. Claims	3 - 3 =	0	X _____ =	00.00
Multiple Dependent Claims			-0- =	00.00

Large Fee Code	Entity Fee ($)	Small Fee Code	Entity Fee ($)	Fee Description
103	22	203	11	Claims in excess of 20
102	82	202	41	Independent claims in excess of 3
104	270	204	135	Multiple dependant claim
108	82	209	41	Reissue independent claims over original patent
110	22	210	11	Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) $790.00

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Fee Code	Entity Fee ($)	Small Fee Code	Entity Fee ($)	Fee Description	Fee Paid
105	130	206	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR prior Examiner action	
115	110	215	55	Extension for reply within first month	
116	400	216	200	Extension for reply within second month	
117	950	217	476	Extension for reply within third month	
118	1,510	218	755	Extension for reply within fourth month	
128	2,060	228	1,030	Extension for reply within fifth month	
119	310	219	155	Notice of Appeal	
120	310	220	155	Filing a brief in support of an appeal	
121	270	221	135	Request for oral hearing	
138	1,510	138	1,610	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,320	241	660	Petition to revive - unintentional	
142	1,320	242	660	Utility issue fee (or reissue)	
143	450	243	226	Design issue fee	
144	670	244	336	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Petitions related to provisional applications	
126	240	126	240	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	790	246	395	Filing a submission after final rejection (37 CFR 1.129(a))	
149	790	249	395	For each additional invention to be examined (37 CFR 1.129(b))	

Other fee (specify)

Other fee (specify)

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($) _____

SUBMITTED BY				Complete *(if applicable)*	
Typed or Printed Name	Eric K. Steffe			Reg. Number	36,688
Signature		Date	March 3, 1998	Deposit Account User ID	

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Divisional application of:	
Jing-Shan HU *et al.*	Art Unit: To Be Assigned
Appl. No. To Be Assigned (Based on prior U.S. Appl. No. 08/469,641, filed June 6, 1995)	Examiner: To Be Assigned
Filed: HEREWITH (March 3, 1998)	Atty. Docket: 1488.1040001/EKS/AJK
For: **Human Vascular Endothelial Growth Factor 3**	

Authorization To Treat A Reply As Incorporating An Extension Of Time Under 37 C.F.R. § 1.136(a)(3)

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

The U.S. Patent and Trademark Office is hereby authorized to treat any concurrent or future reply that requires a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. The U.S. Patent and Trademark Office is hereby authorized to charge all required extension of time fees to our Deposit Account No. 19-0036, if such fees are not otherwise provided for in such reply. A duplicate of this petition is enclosed.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Eric K. Steffe
Attorney for Applicants
Registration No. 36,688

Date: March 3, 1998
1100 New York Avenue, N.W., Suite 600
Washington, D.C. 20005-3934
(202) 371-2600
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SKGF Rev. 1/26/98 clp

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.
ATTORNEYS AT LAW
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WASHINGTON, D.C. 20005-3934

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FACSIMILE (202) 371-2540, (202) 371-6566

WRITER'S DIRECT NUMBER:

INTERNET ADDRESS:

March 3, 1998

Box Patent Application

Assistant Commissioner for Patents
Washington, D.C. 20231

Re: U.S. Divisional Utility Patent Application Under 37 C.F.R. § 1.53(b)
(Based on U.S. Appl. No. 08/469,641, filed: June 6, 1995)
Appl. No. To Be Assigned; Filed: HEREWITH (March 3, 1998)
For: **Human Vascular Endothelial Growth Factor 3**
Inventors: Jing-Shan HU *et al.*
Our Ref: 1488.1040001/EKS/AJK

Sir:

The following documents are forwarded herewith for appropriate action by the U.S. Patent and Trademark Office:

1. Utility Patent Application Transmittal (PTO/SB/05 rev 1);

2. U.S. Divisional Utility Patent Application entitled:

Human Vascular Endothelial Growth Factor 3

and naming as inventors:

Jing-Shan HU
Henrik S. OLSEN, and
Craig A. ROSEN

the application consisting of:

a. A specification containing 49 total pages:

(i) 44 pages of description prior to the claims, including a sequence listing on pages 42 to 44;

(ii) 4 pages of claims (20 claims);

(iii) a one (1) page abstract;

b. 2 sheets of drawings: (Figures 1-2);

c. A copy of the executed Declaration, as filed in U.S. Appl. No. 08/469,651, filed June 6, 1995;

d. Our check No. 21242 for $790.00 to cover:

$790.00 filing fee for patent application;

3. Preliminary Amendment;

4. PTO Fee Transmittal Form PTO/SB/17;

5. Second Preliminary Amendment;

6. Authorization to Treat a Reply As Incorporating An Extension of Time Under 37 C.F.R. § 1.136(a)(3); and

7. Two (2) return postcards.

It is respectfully requested that, of the two attached postcards, one be stamped with the filing date of these documents and returned to our courier, and the other, prepaid postcard, be stamped with the filing date and unofficial application number and returned as soon as possible.

The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 19-0036. A duplicate of this letter is enclosed.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Eric K. Steffe
Attorney for Applicants
Registration No. 36,688

EKS/AJK/neh
Enclosures

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SKGF ver. 2/23/98 mac

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
Hu *et al.*	Art Unit: To be assigned
Appl. No.: To be assigned	Examiner: To be assigned
Filed: Herewith	Atty Docket: 1488.1040001
For: **Human Vascular Endothelial Growth Factor 3**	

Preliminary Amendment

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

In advance of prosecution, please amend the application as follows.

In the Specification

At page 1, after the title, please insert --This application is a divisional of United States Appl. No. 08/469,641, filed June 6, 1995, which disclosure is herein incorporated by reference.--

In the Claims

Please cancel claims 1-10 and 12-20.

Remarks

This amendment contains no new matter. Following entry of this amendment, claim 11 is pending in this application.

The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 19-0036.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Eric K. Steffe
Attorney for Applicants
Registration No. 36,688

Date: March 3, 1998

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Washington, D.C. 20005-3934
(202) 371-2600

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SKGF 1/28/98 dcw

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
Hu *et al.*	Art Unit: To be assigned
Appl. No.: To be assigned	Examiner: To be assigned
Filed: Herewith	Atty Docket: 1488.1040001
For: **Vascular Endothelial Growth Factor 3**	

Second Preliminary Amendment

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

In advance of prosecution, please amend the application as follows.

In the Claims

Please cancel claim 11. Please add the following new claims:

--21. An isolated polypeptide produced by expression of a DNA selected from the group consisting of:

(a) the DNA of SEQ ID NO:1; and

(b) DNA which hybridizes under stringent conditions with the DNA of SEQ ID NO:1 or its complement.

22. An isolated polypeptide comprising at least 30 contiguous amino acids of SEQ ID NO:2.

23. The isolated polypeptide of claim 22, which comprises at least 50 contiguous amino acids of SEQ ID NO:2.--

Remarks

Support for new claims 21-23 can be found, *inter alia*, at pages 9, 12 and 18 of the specification. Thus, no new matter has been added by way of new claims 21-23.

It is believed that new claim 21 may correspond to claim 12 of U.S. Patent No. 5,607,918, issued March 4, 1997.

The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 19-0036.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Eric K. Steffe
Attorney for Applicants
Registration No. 36,688

Date: March 3, 1998

1100 New York Avenue, N.W.
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SKGF 1/28/98 dcw

Human Vascular Endothelial Growth Factor 3

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. The polypeptide of the present invention has been identified as a member of the vascular endothelial growth factor family. More particularly, the polypeptide of the present invention is vascular endothelial growth factor 3, sometimes hereinafter referred to as "VEGF3." The invention also relates to inhibiting the action of such polypeptide.

The formation of new blood vessels, or angiogenesis, is essential for embryonic development, subsequent growth, and tissue repair. Angiogenesis, however, is an essential part of certain pathological conditions such as neoplasia, for example, tumors and gliomas, and abnormal angiogenesis is associated with other diseases such as inflammation, rheumatoid arthritis, psoriasis, and diabetic retinopathy (Folkman, J. and Klagsbrun, M., Science 235:442-447,(1987)).

Both acidic and basic fibroblast growth factor molecules are mitogens for endothelial cells and other cell types. Angiotropin and angiogenin can induce angiogenesis, although their functions are unclear (Folkman, J., 1993, Cancer

Medicine pp. 153-170, Lea and Febiger Press). A highly selective mitogen for vascular endothelial cells is vascular endothelial growth factor or VEGF (Ferrara, N., *et al.*, Endocr. Rev. 13:19-32, (1992)), also known as vascular permeability factor (VPF). Vascular endothelial growth factor is a secreted angiogenic mitogen whose target cell specificity appears to be restricted to vascular endothelial cells.

The murine VEGF gene has been characterized and its expression pattern in embryogenesis has been analyzed. A persistent expression of VEGF was observed in epithelial cells adjacent to fenestrated endothelium, e.g., in choroid plexus and kidney glomeruli. The data was consistent with a role of VEGF as a multifunctional regulator of endothelial cell growth and differentiation (Breier, G. *et al.* Development, 114:521-532 (1992)).

VEGF is structurally related to the α and β chains of platelet-derived growth factor (PDGF), a mitogen for mesenchymal cells and placenta growth factor (PLGF), an endothelial cell mitogen. These three proteins belong to the same family and share a conserved motif. Eight cysteine residues contributing to disulfide-bond formation are strictly conserved in these proteins. Alternatively spliced mRNAs have been identified for both VEGF, PLGF and PDGF and these different splicing products differ in biological activity and in receptor-binding specificity. VEGF and PDGF function as homo-dimers or hetero-dimers and bind to receptors which elicit intrinsic tyrosine kinase activity following receptor dimerization.

VEGF has four different forms of 121, 165, 189 and 206 amino acids due to alternative splicing. VEGF121 and VEGF165 are soluble and are capable of promoting angiogenesis, whereas VEGF189 and VEGF306 are bound to heparin containing proteoglycans in the cell surface. The temporal and spatial expression of VEGF has been correlated with physiological

proliferation of the blood vessels (Gajdusek, C.M., and Carbon, S.J., Cell Physiol., 139:570-579, (1989)); McNeil, P.L., Muthukrishnan, L., Warder, E., D'Amore, P.A., J. Cell. Biol., 109:811-822, (1989)). Its high affinity binding sites are localized only on endothelial cells in tissue sections (Jakeman, L.B., *et al.*, Clin. Invest. 89:244-253, (1989)). The factor can be isolated from pituitary cells and several tumor cell lines, and has been implicated in some human gliomas (Plate, K.H. Nature 359:845-848, (1992)). Interestingly, expression of VEGF121 or VEGF165 confers on Chinese hamster ovary cells the ability to form tumors in nude mice (Ferrara, N., *et al.*, J. Clin. Invest. 91:160-170, (1993)). The inhibition of VEGF function by anti-VEGF monoclonal antibodies was shown to inhibit tumor growth in immune-deficient mice (Kim, K.J., Nature 362:841-844, (1993)). Further, a dominant-negative mutant of the VEGF receptor has been shown to inhibit growth of glioblastomas in mice.

Vascular permeability factor, has also been found to be responsible for persistent microvascular hyperpermeability to plasma proteins even after the cessation of injury, which is a characteristic feature of normal wound healing. This suggests that VPF is an important factor in wound healing. Brown, L.F. *et al.*, J. Exp. Med., 176:1375-9 (1992).

The expression of VEGF is high in vascularized tissues, (e.g., lung, heart, placenta and solid tumors) and correlates with angiogenesis both temporally and spatially. VEGF has also been shown to induce angiogenesis *in vivo*. Since angiogenesis is essential for the repair of normal tissues, especially vascular tissues, VEGF has been proposed for use in promoting vascular tissue repair (e.g., in atherosclerosis).

U.S. Patent No. 5,073,492, issued December 17, 1991 to Chen *et al.*, discloses a method for synergistically enhancing endothelial cell growth in an appropriate environment which

comprises adding to the environment, VEGF, effectors and serum-derived factor. Also, vascular endothelial cell growth factor C sub-unit DNA has been prepared by polymerase chain reaction techniques. The DNA encodes a protein that may exist as either a hetero-dimer or homo-dimer. The protein is a mammalian vascular endothelial cell mitogen and, as such, is useful for the promotion of vascular development and repair, as disclosed in European Patent Application No. 92302750.2, published September 30, 1992.

The polypeptides of the present invention have been putatively identified as a novel vascular endothelial growth factor based on amino acid sequence homology to human VEGF.

In accordance with one aspect of the present invention, there are provided novel mature polypeptides, as well as biologically active and diagnostically or therapeutically useful fragments, analogs and derivatives thereof. The polypeptides of the present invention are of human origin.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding the polypeptides of the present invention, including mRNAs, DNAs, cDNAs, genomic DNA as well as biologically active and diagnostically or therapeutically useful fragments, analogs and derivatives thereof.

In accordance with still another aspect of the present invention, there are provided processes for producing such polypeptides by recombinant techniques comprising culturing recombinant prokaryotic and/or eukaryotic host cells, containing a nucleic acid sequence encoding a polypeptide of the present invention, under conditions promoting expression of said proteins and subsequent recovery of said proteins.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptide, or polynucleotide encoding such polypeptide for therapeutic purposes, for example, to stimulate angiogenesis, wound-healing, and to promote vascular tissue repair.

In accordance with yet another aspect of the present invention, there are provided antibodies against such polypeptides.

In accordance with yet another aspect of the present invention, there are provided antagonists to such polypeptides, which may be used to inhibit the action of such polypeptides, for example, to inhibit the growth of tumors, to treat diabetic retinopathy, inflammation, rheumatoid arthritis and psoriasis.

In accordance with another aspect of the present invention, there are provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to nucleic acid sequences of the present invention.

In accordance with another aspect of the present invention, there are provided methods of diagnosing diseases or a susceptibility to diseases related to mutations in nucleic acid sequences of the present invention and proteins encoded by such nucleic acid sequences.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptides, or polynucleotides encoding such polypeptides, for *in vitro* purposes related to scientific research, synthesis of DNA and manufacture of DNA vectors.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Fig. 1 shows the cDNA sequence and the corresponding deduced amino acid sequence of the polypeptide of the present invention. The standard one letter abbreviations for amino acids are used. Sequencing was performed using 373 Automated DNA Sequencer (Applied Biosystems, Inc.). Sequencing accuracy is predicted to be greater than 97%.

Fig. 2 is an illustration of the amino acid sequence homology between the polypeptide of the present invention and human VEGF.

In accordance with one aspect of the present invention, there are provided isolated nucleic acid molecules (polynucleotides) which encode for the mature polypeptides having the deduced amino acid sequence of Figure 1 (SEQ ID NO:2) or for the mature polypeptide encoded by the cDNA of the clone deposited as ATCC1 Deposit No. ____ on May 26, 1995. A polynucleotide encoding a polypeptide of the present invention may be obtained from early stage human embryo (week 8 to 9) osteoclastomas, adult heart or several breast cancer cell lines. The polynucleotide of this invention was discovered in a cDNA library derived from human colon tissue. It is structurally related to the VEGF/PDGF family. VEGF3 contains an open reading frame encoding a protein of 221 amino acid residues. The protein exhibits the highest amino acid sequence homology to human vascular endothelial growth factor with 36.199 % identity and 66.063 % similarity.

It is particularly important that all eight cysteines are conserved within all the polypeptide of the present invention and the signature for the PDGF/VEGF family, PXCVXXXRCXGCCN, is conserved in VEGF3 (see Figure 2).

The VEGF3 polypeptide of the present invention is meant to include the full length polypeptide and polynucleotide sequence which encodes for any leader sequences and for active fragments of the full length polypeptide. Active fragments are meant to include any portions of the full length amino acid sequence which have less than the full 221 amino acids of the full length amino acid sequence as shown in SEQ ID No. 2 and Figure 1, but still contain the eight cysteine residues shown conserved in Figure 1 and such fragments still contain VEGF3 activity.

The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in Figure 1 (SEQ ID NO:1) or that of the deposited clone or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature polypeptide as the DNA of Figure 1 (SEQ ID NO:1) or the deposited cDNA.

The polynucleotide which encodes for the mature polypeptide of Figure 1 (SEQ ID NO:2) or for the mature polypeptide encoded by the deposited cDNA may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figure 1 (SEQ ID NO:2) or the polypeptide encoded by the cDNA of the deposited clone. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in Figure 1 (SEQ ID NO:2) or the same mature polypeptide encoded by the

cDNA of the deposited clone as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the polypeptide of Figure 1 (SEQ ID NO:2) or the polypeptide encoded by the cDNA of the deposited clone. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figure 1 (SEQ ID NO:1) or of the coding sequence of the deposited clone. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexa-histidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

Fragments of the full length gene of the present invention may be used as a hybridization probe for a cDNA library to isolate the full length cDNA and to isolate other cDNAs which have a high sequence similarity to the gene or

similar biological activity. Probes of this type preferably have at least 30 bases and may contain, for example, 50 or more bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene including regulatory and promotor regions, exons, and introns. An example of a screen comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 70%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which either retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNAs of Figure 1 (SEQ ID NO:1) or the deposited cDNA(s).

Alternatively, the polynucleotide may have at least 20 bases, preferably 30 bases, and more preferably at least 50 bases which hybridize to a polynucleotide of the present invention and which has an identity thereto, as hereinabove described, and which may or may not retain activity. For example, such polynucleotides may be employed as probes for the polynucleotide of SEQ ID NO:1, for example, for recovery

of the polynucleotide or as a diagnostic probe or as a PCR primer.

Thus, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% and more preferably at least a 95% identity to a polynucleotide which encodes the polypeptide of SEQ ID NO:2 as well as fragments thereof, which fragments have at least 30 bases and preferably at least 50 bases and to polypeptides encoded by such polynucleotides.

The deposit(s) referred to herein will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for purposes of Patent Procedure. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The present invention further relates to a polypeptides which have the deduced amino acid sequence of Figure 1 (SEQ ID NO:2) or which has the amino acid sequence encoded by the deposited cDNA, as well as fragments, analogs and derivatives of such polypeptide.

The terms "fragment," "derivative" and "analog" when referring to the polypeptide of Figure 1 (SEQ ID NO:2) or that encoded by the deposited cDNA, means a polypeptide which retains the conserved motif of VEGF proteins as shown in Figure 1 (SEQ ID NO:2) and essentially the same biological function or activity.

The polypeptides of the present invention may be recombinant polypeptides, natural polypeptides or synthetic polypeptides, preferably recombinant polypeptides.

The fragment, derivative or analog of the polypeptide of Figure 1 (SEQ ID NO:2) or that encoded by the deposited cDNA may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide or (v) one in which comprises fewer amino acid residues shown in SEQ ID No. 2 and retains the conserved motif and yet still retains activity characteristic of the VEGF family of polypeptides. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a

composition, and still be isolated in that such vector or composition is not part of its natural environment.

The polypeptides of the present invention include the polypeptide of SEQ ID NO:2 (in particular the mature polypeptide) as well as polypeptides which have at least 70% similarity (preferably at least 70% identity) to the polypeptide of SEQ ID NO:2 and more preferably at least 90% similarity (more preferably at least 90% identity) to the polypeptide of SEQ ID NO:2 and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the polypeptide of SEQ ID NO:2 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide.

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the

form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the VEGF3 genes of the present invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli. lac or trp, the phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator.

The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Streptomyces, Salmonella typhimurium; fungal cells, such as yeast; insect cells such as Drosophila S2 and Spodoptera Sf9; animal cells such as CHO, COS or Bowes melanoma; adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pBluescript SK, pBSKS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO,

pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation. (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with

prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase

with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well know to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of

monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The polypeptides can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

VEGF3 polypeptide may be employed to promote angiogenesis, for example, to stimulate the growth of transplanted tissue where coronary bypass surgery is performed. VEGF3 may also be employed to stimulate wound

healing, particularly to re-vascularize damaged tissues or where new capillary angiogenesis is desired. VEGF3 may be employed to treat full-thickness wounds such as dermal ulcers, including pressure sores, venous ulcers, and diabetic ulcers. In addition, VEGF3 may be employed to treat full-thickness burns and injuries where a skin graft or flap is used to repair such burns and injuries. VEGF3 may also be employed for use in plastic surgery, for example, for the repair of lacerations from trauma and cuts in association with surgery.

Along these same lines, VEGF3 may be employed to induce the growth of damaged bone, periodontium or ligament tissue. VEGF3 may also be employed for regenerating supporting tissues of the teeth, including cementum and periodontal ligament, that have been damaged by disease and trauma.

Since angiogenesis is important in keeping wounds clean and non-infected, VEGF3 may be employed in association with surgery and following the repair of cuts. It may also be employed for the treatment of abdominal wounds where there is a high risk of infection.

VEGF3 may be employed for the promotion of endothelialization in vascular graft surgery. In the case of vascular grafts using either transplanted or synthetic material, VEGF3 can be applied to the surface of the graft or at the junction to promote the growth of vascular endothelial cells. VEGF3 may also be employed to repair damage of myocardial tissue as a result of myocardial infarction. VEGF3 may also be employed to repair the cardiac vascular system after ischemia. VEGF3 may also be employed to treat damaged vascular tissue as a result of coronary artery disease and peripheral and CNS vascular disease.

VEGF3 may also be employed to coat artificial prostheses or natural organs which are to be transplanted in the body to minimize rejection of the transplanted material and to stimulate vascularization of the transplanted materials.

VEGF3 may also be employed for vascular tissue repair, for example, that required during arteriosclerosis and following balloon angioplasty where vascular tissues are damaged.

VEGF3 nucleic acid sequences and VEGF3 polypeptides may also be employed for *in vitro* purposes related to scientific research, synthesis of DNA and manufacture of DNA vectors, and for the production of diagnostics and therapeutics to treat human disease. For example, VEGF3 may be employed for *in vitro* culturing of vascular endothelial cells, where it is added to the conditional medium in a concentration from 10 pg/ml to 10 ng/ml.

This invention provides methods for identification of VEGF3 receptors. The gene encoding the receptor can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting (Coligan, et al., Current Protocols in Immun., 1(2), Chapter 5, (1991)). Preferably, expression cloning is employed wherein polyadenylated RNA is prepared from a cell responsive to VEGF3, and a cDNA library created from this RNA is divided into pools and used to transfect COS cells or other cells that are not responsive to VEGF3. Transfected cells which are grown on glass slides are exposed to labeled VEGF3. VEGF3 can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase. Following fixation and incubation, the slides are subjected to autoradiographic analysis. Positive pools are identified and sub-pools are prepared and retransfected using an iterative sub-pooling and rescreening process, eventually yielding a single clone that encodes the putative receptor.

As an alternative approach for receptor identification, labeled VEGF3 can be photoaffinity linked with cell membrane or extract preparations that express the receptor molecule. Cross-linked material is resolved by PAGE and exposed to X-

ray film. The labeled complex containing VEGF3 is then excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the gene encoding the putative receptor.

This invention is also related to a method of screening compounds to identify those which are VEGF3 agonists or antagonists. An example of such a method takes advantage of the ability of VEGF3 to significantly stimulate the proliferation of human endothelial cells in the presence of the comitogen Con A. Endothelial cells are obtained and cultured in 96-well flat-bottomed culture plates (Costar, Cambridge, MA) in a reaction mixture supplemented with Con-A (Calbiochem, La Jolla, CA). Con-A, polypeptides of the present invention and the compound to be screened are added. After incubation at 37°C, cultures are pulsed with 1 μCi of ^{3}[H]thymidine (5 Ci/mmol; 1 Ci = 37 BGq; NEN) for a sufficient time to incorporate the ^{3}[H] and harvested onto glass fiber filters (Cambridge Technology, Watertown, MA). Mean ^{3}[H]-thymidine incorporation (cpm) of triplicate cultures is determined using a liquid scintillation counter (Beckman Instruments, Irvine, CA). Significant ^{3}[H]thymidine incorporation, as compared to a control assay where the compound is excluded, indicates stimulation of endothelial cell proliferation.

To assay for antagonists, the assay described above is performed and the ability of the compound to inhibit ^{3}[H]thymidine incorporation in the presence of VEGF3 indicates that the compound is an antagonist to VEGF3. Alternatively, VEGF3 antagonists may be detected by combining VEGF3 and a potential antagonist with membrane-bound VEGF3 receptors or recombinant receptors under appropriate conditions for a competitive inhibition assay. VEGF3 can be labeled, such as by radioactivity, such that the number of VEGF3 molecules

bound to the receptor can determine the effectiveness of the potential antagonist.

Alternatively, the response of a known second messenger system following interaction of VEGF3 and receptor would be measured and compared in the presence or absence of the compound. Such second messenger systems include but are not limited to, cAMP guanylate cyclase, ion channels or phosphoinositide hydrolysis. In another method, a mammalian cell or membrane preparation expressing the VEGF3 receptor is incubated with labeled VEGF3 in the presence of the compound. The ability of the compound to enhance or block this interaction could then be measured.

Potential VEGF3 antagonists include an antibody, or in some cases, an oligonucleotide, which bind to the polypeptide and effectively eliminate VEGF3 function. Alternatively, a potential antagonist may be a closely related protein which binds to VEGF3 receptors, however, they are inactive forms of the polypeptide and thereby prevent the action of VEGF3. Examples of these antagonists include a negative dominant mutant of the VEGF3 polypeptide, for example, one chain of the hetero-dimeric form of VEGF3 may be dominant and may be mutated such that biological activity is not retained. An example of a negative dominant mutant includes truncated versions of a dimeric VEGF3 which is capable of interacting with another dimer to form wild type VEGF3, however, the resulting homo-dimer is inactive and fails to exhibit characteristic VEGF activity.

Another potential VEGF3 antagonist is an antisense construct prepared using antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an

antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix -see Lee et al., Nucl. Acids Res., 6:3073 (1979); Cooney et al, Science, 241:456 (1988); and Dervan et al., Science, 251: 1360 (1991)), thereby preventing transcription and the production of VEGF3. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into the VEGF3 polypeptide (Antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of VEGF3.

Potential VEGF3 antagonists also include small molecules which bind to and occupy the active site of the polypeptide thereby making the catalytic site inaccessible to substrate such that normal biological activity is prevented. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

The antagonists may be employed to treat tumors since angiogenesis and neovascularization are essential steps in tumor growth. The mRNA encoding for VEGF3 is found to be expressed at moderate levels in at least two breast tumor cell lines which is indicative of the role of VEGF3 polypeptides in the malignant phenotype. Gliomas are also a type of neoplasia which may be treated with the antagonists of the present invention.

The antagonists may also be used to treat inflammation caused by increased vascular permeability. In addition to these disorders, the antagonists may also be employed to treat diabetic retinopathy, rheumatoid arthritis and psoriasis.

The antagonists may be employed in a composition with a pharmaceutically acceptable carrier, e.g., as hereinafter described.

The VEGF3 polypeptides and agonists and antagonists may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the polypeptide or agonist or antagonist, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the pharmaceutical compositions may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the topical, intravenous, intraperitoneal, intramuscular, intratumor, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, the pharmaceutical compositions are administered in an amount of at least about 10 μg/kg body weight and in most cases they will be administered in an amount not in excess of about 8 mg/Kg body weight per day. In most cases, the dosage is from about 10 μg/kg to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

The VEGF3 polypeptides, and agonists or antagonists which are polypeptides may also be employed in accordance with the present invention by expression of such polypeptide *in vivo*, which is often referred to as "gene therapy."

Thus, for example, cells such as bone marrow cells may be engineered with a polynucleotide (DNA or RNA) encoding for the polypeptide *ex vivo*, the engineered cells are then provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding the polypeptide of the present invention.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo*, for example, by procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding a polypeptide of the present invention may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such methods should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retroviral particle, for example, an adenovirus, which may be used to engineer cells *in vivo* after combination with a suitable delivery vehicle.

Retroviruses from which the retroviral plasmid vectors hereinabove mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., Biotechniques, Vol. 7, No. 9, 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and β-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The nucleic acid sequence encoding the polypeptide of the present invention is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or hetorologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the β-actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, ψ-2, ψ-AM, PA12, T19-14X, VT-19-17-H2, ψCRE, ψCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, Human Gene Therapy, Vol. 1, pgs. 5-14 (1990), which is incorporated herein by reference

in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and $CaPO_4$ precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

This invention is also related to the use of the VEGF3 gene as part of a diagnostic assay for detecting diseases or susceptibility to diseases related to the presence of mutations in VEGF3 nucleic acid sequences.

Individuals carrying mutations in the VEGF3 gene may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki *et al.*, Nature, 324:163-166 (1986)) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid encoding VEGF3 can be used to identify and analyze VEGF3 mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be

identified by hybridizing amplified DNA to radiolabeled VEGF3 RNA or alternatively, radiolabeled VEGF3 antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers *et al.*, Science, 230:1242 (1985)).

Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (e.g., Cotton *et al.*, PNAS, USA, 85:4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., Restriction Fragment Length Polymorphisms (RFLP)) and Southern blotting of genomic DNA.

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

The present invention also relates to a diagnostic assay for detecting altered levels of VEGF3 protein in various tissues since an over-expression of the proteins compared to normal control tissue samples may detect the presence of a disease or susceptibility to a disease, for example, abnormal cellular differentiation. Assays used to detect levels of VEGF3 protein in a sample derived from a host are well-known

to those of skill in the art and include radioimmunoassays, competitive-binding assays, Western Blot analysis, ELISA assays and "sandwich" assay. An ELISA assay (Coligan, et al., Current Protocols in Immunology, 1(2), Chapter 6, (1991)) initially comprises preparing an antibody specific to the VEGF3 antigen, preferably a monoclonal antibody. In addition a reporter antibody is prepared against the monoclonal antibody. To the reporter antibody is attached a detectable reagent such as radioactivity, fluorescence or, in this example, a horseradish peroxidase enzyme. A sample is removed from a host and incubated on a solid support, e.g. a polystyrene dish, that binds the proteins in the sample. Any free protein binding sites on the dish are then covered by incubating with a non-specific protein, such as, bovine serum albumen. Next, the monoclonal antibody is incubated in the dish during which time the monoclonal antibodies attach to any VEGF3 proteins attached to the polystyrene dish. All unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to VEGF3. Unattached reporter antibody is then washed out. Peroxidase substrates are then added to the dish and the amount of color developed in a given time period is a measurement of the amount of VEGF3 protein present in a given volume of patient sample when compared against a standard curve.

A competition assay may be employed wherein antibodies specific to VEGF3 are attached to a solid support. Polypeptides of the present invention are then labeled, for example, by radioactivity, and a sample derived from the host are passed over the solid support and the amount of label detected, for example by liquid scintillation chromatography, can be correlated to a quantity of VEGF3 in the sample.

A "sandwich" assay is similar to an ELISA assay. In a "sandwich" assay VEGF3 is passed over a solid support and

binds to antibody attached to a solid support. A second antibody is then bound to the VEGF3. A third antibody which is labeled and specific to the second antibody is then passed over the solid support and binds to the second antibody and an amount can then be quantified.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphism's) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the cDNA is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60 bases. For a review of this technique, see Verma *et al.*, Human Chromosomes: a Manual of Basic Techniques. Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures

known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptide corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptide into an animal or by administering the polypeptide to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptide itself. In this manner, even a sequence encoding only a fragment of the polypeptide can be used to generate antibodies binding the whole native polypeptide. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, Nature, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, *et al.*, 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic polypeptide products of this invention.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples, certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 μg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 μl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 μg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. *et al.*, Nucleic Acids Res., 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic

oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., *et al.*, Id., p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 μg of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described by the method of Graham, F. and Van der Eb, A., Virology, 52:456-457 (1973).

Example 1

Cloning and expression of VEGF3 using the baculovirus expression system

The DNA contained in the deposited clone which encodes the VEGF3 protein, was amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer has the sequence 5' GCATGGATCCCAGCCTGA TGCCCCTGGCC (SEQ ID NO:4) and contains a BamH1 restriction enzyme site and nucleotide sequence complementary to the 5' sequence of VEGF3 (nt. 150-166).

The 3' primer has the sequence 5' GCATTCTAGACCCTGCTGAG TCTGAAAAGC 3' (SEQ ID NO:5) and contains the cleavage site for the restriction enzyme XbaI and nucleotides complementary to the 3' sequence of VEGF3.

The amplified sequences were isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101, Inc., La Jolla, CA). The fragment was then digested with the endonuclease BamH1 and XbaI and then purified again on a 1% agarose gel. This fragment was ligated to A2GP baculovirus transfer vector with leader peptide sequence (PHarmingen) at the BamH1 and XbaI sites. Through this ligation, VEGF3 cDNA was cloned in frame with the signal sequence of baculovirus

gp67 gene and was located at the 3' end of the signal sequence in the vector. This is designated pA2GP-VEGF3.

To clone VEGF3 with the signal sequence of gp67 gene to the pRG1 vector for expression, VEGF3 with the signal sequence and some upstream sequence were excised from the pA2GP-VEGF3 plasmid at the Xho restriction endonuclease site located upstream of the VEGF3 cDNA and at the XbaI restriction endonuclease site by XhoI and XbaI restriction enzyme. This fragment was separated from the rest of vector on a 1% agarose gel and was purified using "Geneclean" kit. It was designated F2.

The A2PG vector (modification of pVL941 vector) is used for the expression of the VEGF3 protein using the baculovirus expression system (for review see: Summers, M.D. and Smith, G.E. 1987, A manual of methods for baculovirus vectors and insect cell culture procedures, Texas Agricultural Experimental Station Bulletin No. 1555). This expression vector contains the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcMNPV) followed by the recognition sites for the restriction endonucleases BamH1, Sma1, XbaI, BglII and Asp718. A site for restriction endonuclease Xho1 is located upstream of BamH1 site. The sequence between Xho1 and BamHI is the same as that in pAcGp67A vector. The polyadenylation site of the simian virus (SV)40 is used for efficient polyadenylation. For an easy selection of recombinant virus the beta-galactosidase gene from E.coli is inserted in the same orientation as the polyhedrin promoter followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences are flanked at both sides by viral sequences for the cell-mediated homologous recombination of cotransfected wild-type viral DNA. Many other baculovirus vectors could be used in place of A2GP such as pRG1, pAc373, pVL941 and pAcIM1 (Luckow, V.A. and Summers, M.D., Virology, 170:31-39).

The plasmid was digested with the restriction enzymes XboI and XbaI and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The DNA was then isolated from a 1% agarose gel using the commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated V2.

Fragment F2 and the dephosphorylated plasmid V2 were ligated with T4 DNA ligase. **E.coli** HB101 cells were then transformed and bacteria identified that contained the plasmid (pBac gp67-VEGF3) with the VEGF3 gene using the enzymes BamH1 and XbaI. The sequence of the cloned fragment was confirmed by DNA sequencing.

5 µg of the plasmid pBac A2GP-VEGF3 was cotransfected with 1.0 µg of a commercially available linearized baculovirus ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA.) using the lipofection method (Felgner et al. Proc. Natl. Acad. Sci. USA, 84:7413-7417 (1987)).

1µg of BaculoGold™ virus DNA and 5 µg of the plasmid pBac A2GP-VEGF3 were mixed in a sterile well of a microtiter plate containing 50 µl of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10 µl Lipofectin plus 90 µl Grace's medium were added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture was added dropwise to the Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate was rocked back and forth to mix the newly added solution. The plate was then incubated for 5 hours at 27°C. After 5 hours the transfection solution was removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum was added. The plate was put back into an incubator and cultivation continued at 27°C for four days.

After four days the supernatant was collected and a plaque assay performed similar as described by Summers and Smith (supra). As a modification an agarose gel with "Blue

Gal" (Life Technologies Inc., Gaithersburg) was used which allows an easy isolation of blue stained plaques. (A detailed description of a "plaque assay" can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after the serial dilution, the virus was added to the cells, blue stained plaques were picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses was then resuspended in an Eppendorf tube containing 200 µl of Grace's medium. The agar was removed by a brief centrifugation and the supernatant containing the recombinant baculovirus was used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes were harvested and then stored at 4°C.

Sf9 cells were grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells were infected with the recombinant baculovirus V-A2GP-VEGF3 at a multiplicity of infection (MOI) of 1. Six hours later the medium was removed and replaced with SF900 II medium minus methionine and cysteine (Life Technologies Inc., Gaithersburg). 42 hours later 5 µCi of ^{35}S-methionine and 5 µCi ^{35}S cysteine (Amersham) were added. The cells were further incubated for 16 hours before they were harvested by centrifugation and the labelled proteins visualized by SDS-PAGE and autoradiography.

Protein from the medium and cytoplasm of the Sf9 cells was analyzed by SDS-PAGE under reducing and non-reducing conditions. The medium was dialyzed against 50 mM MES, pH 5.8. Precpitates were obtained after dialysis and resuspended in 100 mM NaCitrate, pH 5.0. The resuspended precipitate was analyzed again by SDS-PAGE and was stained with Coomassie Brilliant Blue.

The medium supernatant was also diluted 1:10 in 50 mM MES, pH 5.8 and applied to an SP-650M column (1.0 x 6.6 cm, Toyopearl) at a flow rate of 1 ml/min. Protein was eluted

with step gradients at 200, 300 and 500 mM NaCl. The VEGF3 was obtained using the elution at 500 mM. The eluate was analyzed by SDS-PAGE in the presence or absence of reducing agent, β-mercaptoethanol and stained by Coommassie Brilliant Blue.

Example 2

Expression via Gene Therapy

Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin, is added. This is then incubated at 37°C for approximately one week. At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

pMV-7 (Kirschmeier, P.T. et al, DNA, 7:219-25 (1988) flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding a polypeptide of the present invention is amplified using PCR primers which correspond to the 5' and 3' end sequences respectively. The 5' primer contains an EcoRI site and the 3' primer includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are

added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is used to transform bacteria HB101, which are then plated onto agar-containing kanamycin for the purpose of confirming that the vector had the gene of interest properly inserted.

The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the gene is then added to the media and the packaging cells are transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his.

The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product.

Example 3

Bacterial Expression and Purification of VEGF3

The DNA of deposited clone which encodes VEGF3, is initially amplified using PCR oligonucleotide primers corresponding to the 5' sequences of the processed VEGF3 protein (minus the signal peptide sequence) and the vector sequences 3' to the VEGF3 gene. Additional nucleotides corresponding to VEGF3 were added to the 5' and 3' sequences respectively. The 5' oligonucleotide primer has the sequence 5' GACTGCATGCACCAGA
GGAAAGTGGTGTC (SEQ ID NO:6) contains a SphI restriction enzyme site followed by VEGF3 coding sequence starting from the presumed terminal amino acid of the processed protein codon. The 3' sequence 5' GACTAGATCTCCTTCGCAGCTTCCGGCAC 3' (SEQ ID NO:7) contains complementary sequences to BglII site located 3' to the VEGF3 DNA insert. The restriction enzyme sites correspond to the restriction enzyme sites on the bacterial expression vector pQE-70 (Qiagen, Inc. 9259 Eton Avenue, Chatsworth, CA, 91311). pQE-70 encodes antibiotic resistance (Amp^r), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites. pQE-9 was then digested with SphI and BglII. The amplified sequences were ligated into pQE-70 and were inserted in frame with the sequence encoding for the histidine tag and the RBS. The ligation mixture was then used to transform E. coli strain M15/rep 4 (Qiagen, Inc.) by the procedure described in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989). M15/rep4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan^r). Transformants are identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies were selected. Plasmid DNA was isolated and confirmed by restriction analysis. Clones containing the desired constructs were grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25

ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells were grown to an optical density 600 ($O.D._{600}$) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") was then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells were grown an extra 3 to 4 hours. Cells were then harvested by centrifugation. The cell pellet was solubilized in the chaotropic agent 6 Molar Guanidine HCl. After clarification, solubilized VEGF3 was purified from this solution by chromatography on a Nickel-Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag (Hochuli, E. et al., J. Chromatography 411:177-184 (1984)). VEGF3 was eluted from the column in 6 molar guanidine HCl pH 5.0 and for the purpose of renaturation adjusted to 3 molar guanidine HCl, 100mM sodium phosphate, 10 mmolar glutathione (reduced) and 2 mmolar glutathione (oxidized). After incubation in this solution for 12 hours the protein was dialyzed to 10 mmolar sodium phosphate.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Olsen, et al.

(ii) TITLE OF INVENTION: Vascular Endothelial Growth Factor 3

(iii) NUMBER OF SEQUENCES:

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: CARELLA, BYRNE, BAIN, GILFILLAN, CECCHI, STEWART & OLSTEIN

(B) STREET: 6 BECKER FARM ROAD

(C) CITY: ROSELAND

(D) STATE: NEW JERSEY

(E) COUNTRY: USA

(F) ZIP: 07068

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5 INCH DISKETTE

(B) COMPUTER: IBM PS/2

(C) OPERATING SYSTEM: MS-DOS

(D) SOFTWARE: WORD PERFECT 5.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: concurrently

(C) CLASSIFICATION:

(vii) ATTORNEY/AGENT INFORMATION:

(A) NAME: FERRARO, GREGORY D.

(B) REGISTRATION NUMBER: 36,134

(C) REFERENCE/DOCKET NUMBER: 325800-

(viii) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 201-994-1700

(B) TELEFAX: 201-994-1744

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 666 BASE PAIRS
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATGAGAAGGT GTAGAATAAG TGGGAGGCCC CCGGCGCCCC CCGGTGTCCC CGCCCAGGCC    60
CCTGTCTCCC AGCCTGATGC CCCTGGCCAC CAGAGGAAAG TGGTGTCATG GATAGATGTG   120
TATACTCGCG CTACCTGCCA GCCCCGGGAG GTGGTGGTGC CCTTGACTGT GGAGCTCATG   180
GGCACCGTGG CCAAACAGCT GGTGCCCAGC TGCGTGACTG TGCAGCGCTG TGGTGGCTGC   240
TGCCCTGACG ATGGCCTGGA GTGTGTGCCC ACTGGGCAGC ACCAAGTCCG GATGCAGATC   300
CTCATGATCC GGTACCCGAG CAGTCAGCTG GGGGAGATGT CCCTGGAAGA ACACAGCCAG   360
TGTGAATGCA GACCTAAAAA AAAGGACAGT GCTGTGAAGC CAGACAGGGC TGCTACTCCC   420
CACCACCGTC CCCAGCCCCG TTCTGTTCCG GGCTGGGACT CTGCCCCCGG AGCACCCTCC   480
CCAGCTGACA TCACCCAATC CCACTCCAGC CCCAGGCCCC TCTGCCCACG CTGCACCCAG   540
CACCACCAGT GCCCTGACCC CCGGACCTGC CGCTGCCGCT GTCGACGCCG CAGCTTCCTC   600
CGTTGTCAAG GGCGGGGCTT AGAGCTCAAC CCAGACACCT GCAGGTGCCG GAAGCTGCGA   660
AGGTGA                                                              666
```

(2) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 221 AMINO ACIDS
(B) TYPE: AMINO ACID
(C) STRANDEDNESS:
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Arg Arg Cys Arg Ile Ser Gly Arg Pro Pro Ala Pro Pro Gly
5 10 15
Val Pro Ala Gln Ala Pro Val Ser Gln Pro Asp Ala Pro Gly His
20 25 30
Gln Arg Lys Val Val Ser Trp Ile Asp Val Tyr Thr Arg Ala Thr
35 40 45
Cys Gln Pro Arg Glu Val Val Val Pro Leu Thr Val Glu Leu Met
50 55 60
Gly Thr Val Ala Lys Gln Leu Val Pro Ser Cys Val Thr Val Gln
65 70 75
Arg Cys Gly Gly Cys Cys Pro Asp Asp Gly Leu Glu Cys Val Pro
80 85 90
Thr Gly Gln His Gln Val Arg Met Gln Ile Leu Met Ile Arg Tyr
95 100 105
Pro Ser Ser Gln Leu Gly Glu Met Ser Leu Glu Glu His Ser Gln
110 115 120
Cys Glu Cys Arg Pro Lys Lys Lys Asp Ser Ala Val Lys Pro Asp
125 130 135
Arg Ala Ala Thr Pro His His Arg Pro Gln Pro Arg Ser Val Pro
140 145 150
Gly Trp Asp Ser Ala Pro Gly Ala Pro Ser Pro Ala Asp Ile Thr
155 160 165
Gln Ser His Ser Ser Pro Arg Pro Leu Cys Pro Arg Cys Thr Gln
170 175 180
His His Gln Cys Pro Asp Pro Arg Thr Cys Arg Cys Arg Cys Arg
185 190 195
Arg Arg Ser Phe Leu Arg Cys Gln Gly Arg Gly Leu Glu Leu Asn
200 205 210
Pro Asp Thr Cys Arg Cys Arg Lys Leu Arg Arg
215 220

WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising a member selected from the group consisting of:

(a) a polynucleotide encoding the polypeptide as set forth in SEQ ID NO:2;

(b) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a); and

(c) a polynucleotide fragment of the polynucleotide of (a) or (b).

2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.

3. The polynucleotide of Claim 2 which encodes the polypeptide as set forth in SEQ ID NO:2.

4. The polynucleotide of Claim 2 which encodes the polypeptide as set forth in SEQ ID NO:2.

5. The polynucleotide of Claim 2 which encodes the polypeptide as set forth in SEQ ID NO:2.

6. An isolated polynucleotide comprising a member selected from the group consisting of:

(a) a polynucleotide which encodes a mature polypeptide encoded by the DNA contained in the deposited clone;

(b) a polynucleotide which encodes a polypeptide expressed by the DNA contained in the deposited clone;

(c) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a) or (b); and

(d) a polynucleotide fragment of the polynucleotide of (a), (b) or (c).

7. A vector containing the DNA of Claim 2.

8. A host cell genetically engineered with the vector of Claim 7.

9. A process for producing a polypeptide comprising: expressing from the host cell of Claim 8 the polypeptide encoded by said DNA.

10. A process for producing cells capable of expressing a polypeptide comprising transforming or transfecting the cells with the vector of Claim 7.

11. A polypeptide selected from the group consisting of (i) a polypeptide having the deduced amino acid sequence of SEQ ID NO:2 and fragments, analogs and derivatives thereof; (ii) a polypeptide comprising amino acid 1 to amino acid 221 of SEQ ID NO:2; and (iii) a polypeptide encoded by the cDNA of the deposited clone and fragments, analogs and derivatives of said polypeptide.

12. A compound effective as an agonist for the polypeptide of claim 11.

13. A compound effective as an antagonist against the polypeptide of claim 11.

14. A method for the treatment of a patient having need of PGSG-1 comprising: administering to the patient a therapeutically effective amount of the polypeptide of claim 11.

15. The method of Claim 14 wherein said therapeutically effective amount of the polypeptide is

administered by providing to the patient DNA encoding said polypeptide and expressing said polypeptide *in vivo*.

16. A method for the treatment of a patient having need of VEGF3 comprising: administering to the patient a therapeutically effective amount of the compound of claim 12.

17. A method for the treatment of a patient having need to inhibit VEGF3 comprising: administering to the patient a therapeutically effective amount of the antagonist of Claim 13.

18. A process for diagnosing a disease or a susceptibility to a disease related to expression of the polypeptide of claim 11 comprising:

determining a mutation in the nucleic acid sequence encoding said polypeptide.

19. A diagnostic process comprising:

analyzing for the presence of the polypeptide of claim 11 in a sample derived from a host.

20. A method for identifying compounds which bind to and activate or inhibit a receptor for the polypeptide of claim 11 comprising:

contacting a cell expressing on the surface thereof a receptor for the polypeptide, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said receptor, with a compound to be screened under conditions to permit binding to the receptor; and

determining whether the compound binds to and activates or inhibits the receptor by detecting the

presence or absence of a signal generated from the interaction of the compound with the receptor.

ABSTRACT

Disclosed is a human VEGF3 polypeptide and DNA(RNA) encoding such VEGF3 polypeptides. Also provided is a procedure for producing such polypeptide by recombinant techniques and antibodies and antagonist against such polypeptide. Also disclosed is a method of using such polypeptide for stimulating wound healing and for vascular tissue repair. Also provided are methods of using the antagonists to inhibit tumor growth, inflammation and to treat diabetic retinopathy, rheumatoid arthritis and psoriasis. Diagnostic methods for detecting mutations in the VEGF3 coding sequence and alterations in the concentration of VEGF3 protein in a sample derived from a host are also disclosed.

ATGAGAAGGTGTAGAATAAGTGGGAGGCCCCCGGCGCCCCCGGTGTCCCCGCCCAGGCC
MetArgArgCysArgIleSerGlyArgProProAlaProProGlyValProAlaGlnAla

CCTGTCTCCCAGCCTGATGCCCCTGGCCACCAGAGGAAAGTGGTGTCATGGATAGATGTG
ProValSerGlnProAspAlaProGlyHisGlnArgLysValValSerTrpIleAspVal

TATACTCGCGCTACCTGCCAGCCCCGGGAGGTGGTGGTGCCCTTGACTGTGGAGCTCATG
TyrThrArgAlaThrCysGlnProArgGluValValValProLeuThrValGluLeuMet

GGCACCGTGGCCAAACAGCTGGTGCCCAGCTGCGTGACTGTGCAGCGCTGTGGTGGCTGC
GlyThrValAlaLysGlnLeuValProSerCysValThrValGlnArgCysGlyGlyCys

TGCCCTGACGATGGCCTGGAGTGTGTGCCCACTGGGCAGCACCAAGTCCGGATGCAGATC
CysProAspAspGlyLeuGluCysValProThrGlyGlnHisGlnValArgMetGlnIle

CTCATGATCCGGTACCCGAGCAGTCAGCTGGGGGAGATGTCCCTGGAAGAACACAGCCAG
LeuMetIleArgTyrProSerSerGlnLeuGlyGluMetSerLeuGluGluHisSerGln

TGTGAATGCAGACCTAAAAAAAAGGACAGTGCTGTGAAGCCAGACAGGGCTGCTACTCCC
CysGluCysArgProLysLysLysAspSerAlaValLysProAspArgAlaAlaThrPro

CACCACCGTCCCCAGCCCCGTTCTGTTCCGGGCTGGGACTCTGCCCCCGGAGCACCCTCC
HisHisArgProGlnProArgSerValProGlyTrpAspSerAlaProGlyAlaProSer

CCAGCTGACATCACCCAATCCCACTCCAGCCCCAGGCCCCTCTGCCCACGCTGCACCCAG
ProAlaAspIleThrGlnSerHisSerSerProArgProLeuCysProArgCysThrGln

CACCACCAGTGCCCTGACCCCCGGACCTGCCGCTGCCGCTGTCGACGCCGCAGCTTCCTC
HisHisGlnCysProAspProArgThrCysArgCysArgCysArgArgArgSerPheLeu

CGTTGTCAAGGGCGGGGCTTAGAGCTCAACCCAGACACCTGCAGGTGCCGGAAGCTGCGA
ArgCysGlnGlyArgGlyLeuGluLeuAsnProAspThrCysArgCysArgLysLeuArg

AGGTGA
ArgEnd

FIGURE 1 1/1

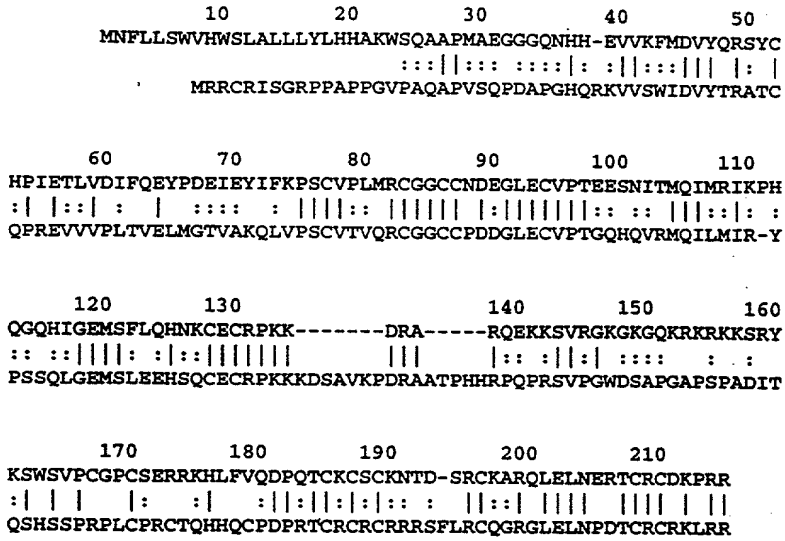

Figure 2. Sequence alignment of VEGF3(lower line) compared to VEGF from human (upper line).

FIGURE 2 1/1

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed bel of the subject matter which is claimed and for which a patent is sought on the invention entitled:

HUMAN VASCULAR ENDOTHELIAL GROWTH FACTOR 3

the specification of which [ ] is attached hereto or [X] was filed on 6/6/95 as Application Serial No. 08/469,641 and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendn referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Fed Regulations, Section 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate li below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on w priority is claimed. Prior Foreign Application(s):

(Number)	(Country)	(Day/Month/Year Filed)	Priority Clai Yes	No
			☐	☐

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject m of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, Un States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) w occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status - patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status - patented, pending, abandoned)

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office conne therewith: John N. Bain (Reg. No. 18,651); John G. Gilfillan, III (Reg. No. 22,746); Elliot M. Olstein (Reg. No. 24,025); Raymond J. Lillie (Reg. 31,778); Charles J. Herron (Reg. No. 28,019); William Squire (Reg. No. 25,378); and Gregory Ferraro (Reg. No. 36,134) of Carella, Bryne, Bain, Gilf Cecchi, Stewart & Olstein; Robert H. Benson (Reg. No. 30,446) of Human Genome Sciences, Inc., 9410 Key West Ave, Rockville, Maryland 20850-3 Address correspondence and telephone calls to Elliot M. Olstein, Esq., c/o Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm R Roseland, NJ 07068 - (201) 994-1700.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonn or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application o patent issued thereon.

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